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Production of Tumor Necrosis Factor and Interleukin-1 by Macrophages from Human Atheromatous Plaques

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The production of cytokines by atheromatous plaque macrophages from human endarterectomy tissue was assessed* in vitro *by short-term cell culture and* in situ *by immunohistology. Macrophages were isolated from plaques of 14 patients undergoing carotid endarterectomy and 7 patients undergoing reconstructive procedures on atheromatous distal aortic and femoral arteries. Tumor necrosis factor (TNF) and interleukin 1 (IL-1) production by plaque macrophages and blood monocytes isolated concurrently from these patients was assessed. TNF release by macrophages from carotid plaques (0.39 ± 0.12 ng/10^6 cells/24 hours) was significantly augmented compared to the release by corresponding blood monocytes (0.014 ± 0.011 ng/10^6 cells/24 hours, P = 0.03), and by macrophages from noncarotid lesions (0.038 ± 0.036 ng/10^6 cells/24 hours, P < 0.04). Cellular TNF expression by macrophages within carotid plaques was also more prominent than in noncarotid lesions. By contrast, IL-1 production by plaque macrophages from both carotid and noncarotid plaques was not augmented compared to blood monocytes, and only infrequent and low-intensity labeling for IL-1 was present on macrophages within plaques from either group. These results provide functional and immunohistological evidence for increased production of TNF but not IL-1 by activated macrophages, indicating local and selective augmention of cytokine production within carotid plaques. This suggests that macrophages play an active role in the inflammatory response within atheromatous carotid plaques. (Am J Pathol 1993, 142:1721–1728)

Atherosclerotic plaques demonstrate many features of acute and chronic inflammation. These include mononuclear inflammatory cell accumulation, degeneration of structural matrix, cell necrosis, proliferation of adjacent cellular components, fibrin deposition, and fibrosis. Macrophages are a prominent component of the inflammatory cell infiltrate in plaques.[1–7] They have a variety of functional capacities that give them the potential to play a pivotal role in the development of atherosclerosis.

Previous studies have demonstrated that macrophages from carotid plaques exhibit augmented tissue factor expression compared to blood monocytes from the same patients.[8] Macrophages in carotid plaques have also been demonstrated to contain mRNA for tissue factor by *in situ* hydridization.[9] This suggests that these macrophages are locally activated and may promote fibrin deposition on ulcerated carotid lesions.

The production of cytokines by plaque macrophages may be important in the initiation and amplification of inflammation in atheroma. In particular, tumor necrosis factor (TNF) and interleukin-1 (IL-1) have the capacity to alter endothelial cell function[10,11] by promoting the expression of procoagulant molecules and inhibiting fibrinolysis. IL-1 can stimulate the proliferation and release of lymphokines from T lymphocytes[12] (which are also prominent in plaques) and may play a role in the recruitment of further macrophages. TNF may contribute to local cell necrosis and promote angiogenesis.[13] Both cytokines have the potential to stimulate fibroblast mitogenesis[12,14,15] and may also contribute to the proliferation of other cells (eg, smooth muscle cells) adjacent to the inflammatory focus.

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In the current studies, the spontaneous production of TNF and IL-1 by macrophages isolated from atheromatous plaques from carotids and other vascular sites was assessed in short-term cell culture. *In situ* cellular expression of these cytokines by macrophages within plaques was assessed by immunoperoxidase staining.

Materials and Methods

Tissues

Endarterectomy tissue was collected from 14 patients undergoing carotid endarterectomy procedures for symptomatic or asymptomatic carotid stenosis and seven patients undergoing reconstructive procedures for atherosclerotic vascular diseases including infrarenal abdominal aortic aneurysms ($n = 4$) and femoral artery disease ($n = 3$). Tissue was collected into sterile normal saline and used immediately for macrophage isolation.

Isolation for Atheromatous Macrophages

Atheromatous tissue was teased apart in culture medium (Eagle's minimal essential medium, Flow Laboratories, Melbourne, Australia) without added serum using sterile 19-gauge hypodermic needles and then by scraping with a sterile scalpel blade. This material was washed through a stainless steel mesh to remove large particulate debris.

The cells were allowed to settle in 50-mm-diameter plastic Petri dishes (Disposable Products, Adelaide, Australia), and after 1 hour, nonadherent cells were washed off with culture medium after agitation. The remaining cells were washed a further two times, removed by 3-minute exposure to trypsin-versene solution (Commonwealth Serum Laboratories, Parkville, Victoria, Australia), and washed twice with culture medium to remove trypsin.

Cells were counted in a Neubauer hemocytometer, and cytospin preparations (Shandon Cytospin II, Shandon Instruments, Runcorn, England) were prepared for morphological and histochemical analysis. Cell morphology was assessed on preparations stained with hematoxylin and eosin. Nonspecific esterase staining was performed by the method of Yam et al.[16]

Isolation of Blood Monocytes

Venous blood (15 ml) was collected from each patient into EDTA anticoagulant during their surgical procedure. Mononuclear cells were prepared by density centrifugation on Lymphopaque (Nyegaard and Company, Oslo, Norway).[17] These cells were allowed to adhere to plastic tissue culture flasks for 1 hour. Adherent mononuclear cells were harvested in the same manner as plaque macrophages and characterized by their nuclear morphology and nonspecific esterase histocytochemistry as described above.

Cytokine Production by Macrophages and Monocytes

Freshly isolated plaque macrophages and blood monocytes were cultured in Eagle's medium at 37 C in a 5% CO_2/air atmosphere for 24 hours. Plaque macrophages were cultured at a concentration of $6.0 \pm 1.4 \times 10^5$ cells/ml (mean ± SEM), and blood monocytes were cultured at $9.6 \pm \times 2.0 \times 10^5$/ml. Supernatants were collected and stored at −70 C. The endotoxin content of culture supernatants was measured using the *Limulus* amebocyte lysate assay (Whittaker MA, Bioproducts, Inc., Walkersville, MD) and was less than 0.05 ng/ml.

After culture, macrophages and monocytes were removed as previously described and stored at −70 C in fresh culture medium. Lysates were prepared immediately prior to assay for TNF and IL-1 by thawing and sonication with 10 1-second pulses, using a Sonifier cell disruptor (Branson Sonic Power Company, Danbury, CT). Phenylmethylsulforyl-fluoride (Sigma), 5 μl of 100 mmol/L solution, was added to each lysate to block any proteinase activity.

TNF in culture supernatants and cell lysates was assayed by enzyme-linked immunosorbent assay (ELISA) (Endogen, Inc., Boston, MA) using a human TNF standard and a monoclonal anti-TNF primary antibody linked to polystyrene plates. Standards were diluted in tissue culture medium, and sample and standards were assayed in duplicate, strictly according to the manufacturer's instructions. Plates were read on a Titerkek Multiskan MC ELISA plate reader. This assay has a sensitivity of less than 50 pg/ml.

To determine whether macrophages may have been activated by exposure to cell debris or other material within the necrotic core of the plaque, freshly isolated normal blood monocytes were cultured at 1×10^6 cell/ml with debris from three carotid plaques, at 37 C. Culture supernatants were collected and assayed for TNF by ELISA. TNF production was undetectable in all three supernatants

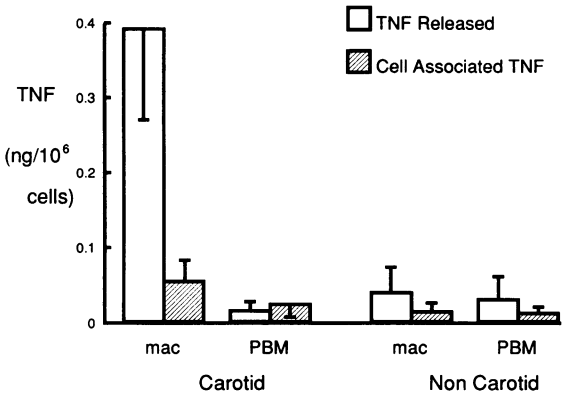


Figure 1. *TNF production by atheromatous macrophages (mac) from carotid and noncarotid plaques and blood monocytes (PBM) from the same patients, showing TNF released in culture (ng/10⁶ cells/24 hours) (open bars) and cell-associated TNF (ng/10⁶ cells) (hatched bars).*

after 1 hour. After 24 hours, TNF production was undetectable in two supernatants and 0.3 ng/10^6 cells/24 hour in the third.

IL-1 in culture supernatants and cell lysates was assayed by ELISA (Cistron Biotechnology, Pine Brook, NJ) in duplicate according to their high-sensitivity 6-hour protocol. The lower limit of sensitivity of this assay is less than 20 pg/ml.

Twenty-four-hour TNF and IL-1 production was determined from the culture supernatant concentration by correcting for the cell concentration in the initial culture. Cellular TNF and IL-1 content was determined from the lysate cytokine concentration by correcting for the cell numbers in each lysate.

Localization of TNF and IL-1 within Atheromatous Plaques

Portions of surgical specimens of seven carotid and seven noncarotid plaques were quick-frozen in liquid nitrogen-chilled isopentane and stored at −70 C. Immunoperoxidase labeling of acetone-fixed, serial cryostat sections (4-μm thickness) was performed with monoclonal antibodies to TNF (Genzyme, Boston, MA), IL-1 (Genzyme), macrophages (CD14, Dako, Sydney, NSW, Australia), smooth muscle cells (smooth muscle cell actin, HHF35, BioGenics Laboratories), and isotype-matched control monoclonal antibodies (Coulter Immunology, Hialeah, FL), as previously described.[18] The results of labeling were assessed by a pathologist without knowledge of the source of the specimen or the results of cytokine bioassays, and graded from 0 to 3+ according to the intensity of cellular staining. Cytospin preparations of lipopolysaccharide stimulated human monocytes (overnight, 10 μg/ml) were used as positive controls for cytokine labeling experiments, and the specificity of labeling was shown in preliminary studies involving quenching of labeling by prior absorption of antibodies with recombinant cytokines.[18]

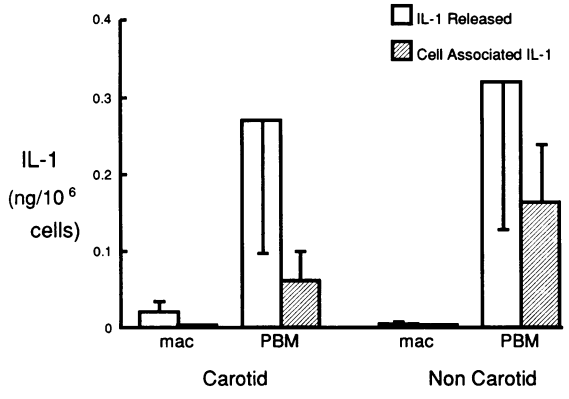


Figure 2. *IL-1 production by atheromatous macrophages (mac) from carotid and noncarotid plaques and blood monocytes (PBM) from the same patients, showing IL-1 released in culture (ng/10⁶ cells/24 hours) (open bars) and cell-associated IL-1 (ng/10⁶ cells) (hatched bars).*

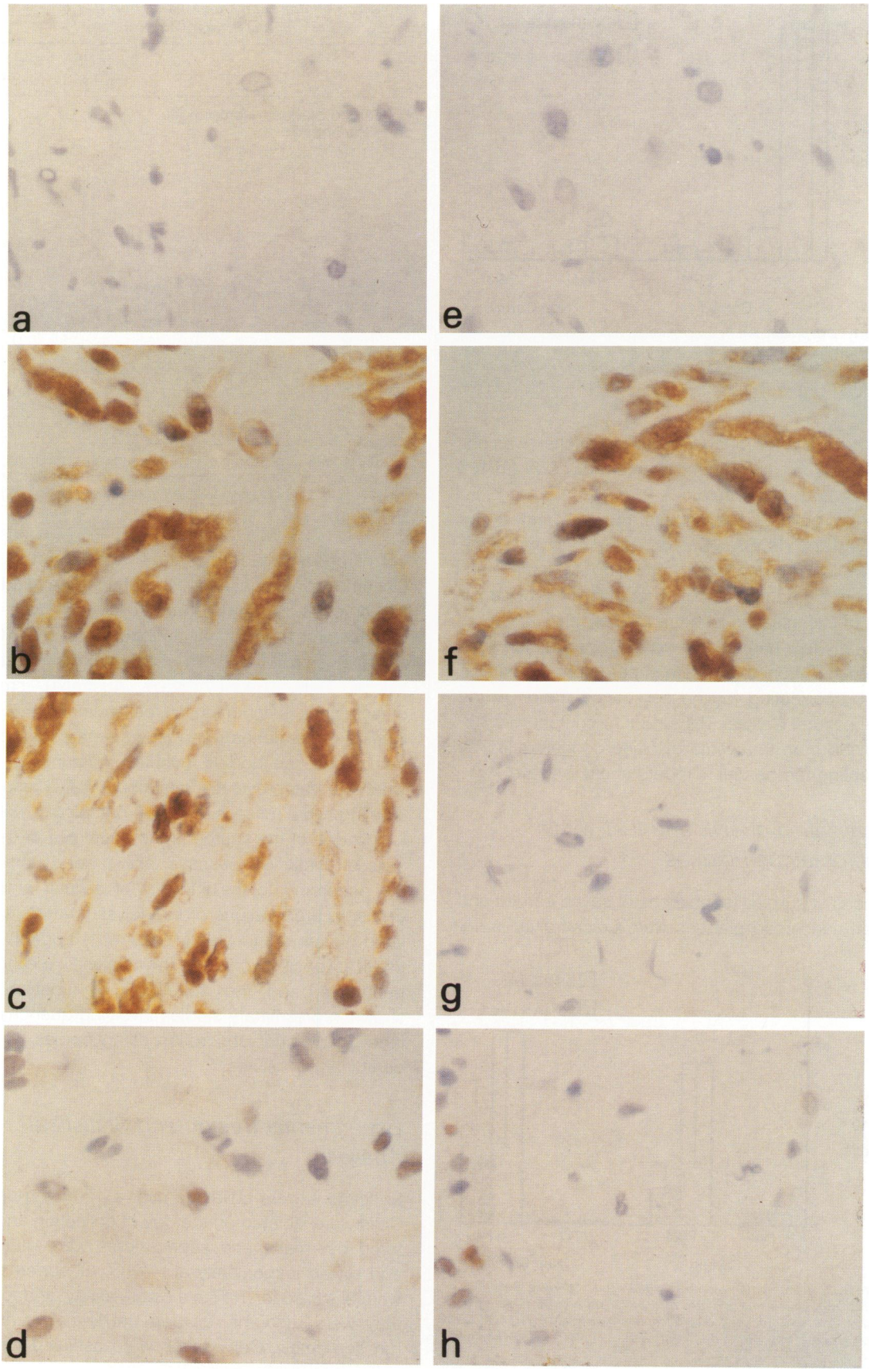
Table 1. *Cellular Expression of TNF and IL-1 by Atheromatous Macrophages* in Situ

	TNF (0–3+)	IL-1 (0–3+)
Carotid plaque macrophages		
Patient no.		
1	+*	0
2	+	0
3	3+	+
4	2+	0
5	3+	+
6	3+	0
7	2+	0
Noncarotid plaque macrophages		
Patient no.		
1	2+	0
2	+	0
3	0	0
4	0	0
5	+	0
6	0	2+
7	+	0

* 0 indicates background staining, 3+ indicates maximum intensity staining equivalent to lipopolysaccharide-stimulated monocytes, + and 2+ indicate increasing intensity of staining, but less than 3+.

Experimental Design and Statistical Analysis

Specimens were collected over a 12-month period. Cells were isolated on the day of collection and cultured overnight, and supernatants were aliquoted and stored immediately at −70 C. All supernatants were assayed for TNF in a single assay, and for IL-1 they were randomly divided between two assays run separately. Cell lysates were assayed for TNF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and IL-1 in single assays. Results are expressed as mean ± the SEM. Statistical significance was assessed by Student's unpaired *t*-test.

Results

Patients

Specimens were collected from 14 patients who underwent carotid endarterectomy. This group comprised nine males and five females of mean age 64.9 ± 2.4 years. Seven noncarotid specimens comprised tissue from four aortic aneurysms and three femoral artery procedures, from seven patients (four males and three females) of mean age 61.1 ± 2.9 years.

Macrophage Isolation

Carotid plaques yielded a mean of $0.4 \pm 0.1 \times 10^6$ cells/g wet weight of tissue. Noncarotid specimens yielded $0.8 \pm 0.3 \times 10^6$ cells/g net weight. After isolation adherent cells were >95% mononuclear. Viability was >90% as determined by Trypan blue exclusion. Esterase staining of selected specimens confirmed >80% esterase positivity, which is consistent with previous results using this technique for isolation of plaque macrophages.

TNF Production by Plaque Macrophages

Macrophages isolated from carotid plaques spontaneously produced TNF in culture (Figure 1). Carotid plaque macrophage TNF production was 0.39 ± 0.12 ng/10^6 cells/24 hours (n = 12). This was significantly greater than TNF production by blood monocytes from the same patients (0.014 ± 0.011 ng/10^6 cells/24 hours; n = 10, P = 0.03) and plaque macrophages from noncarotid lesions (0.038 ± 0.036 ng/10^6 cells/24 hours; n = 6, $P < 0.04$). TNF production by blood monocytes from patients with noncarotid lesions (0.029 ± 0.032 ng/10^6 cells/24 hours; n = 6) was not significantly different from plaque macrophage TNF production in these patients.

TNF was detectable in cell lysates of macrophages and monocytes from some patients undergoing endarterectomy. Carotid plaque macrophages contained 0.053 ± 0.028 ng/10^6 cells of TNF (n = 14). Blood monocytes from these patients contained 0.023 ± 0.019 ng/10^6 cells (n = 14). Plaque macrophages from noncarotid lesions contained 0.013 ± 0.014 ng/10^6 cells (n = 6), and blood monocytes from these patients contained 0.011 ± 0.011 ng/10^6 cells (n = 6).

IL-1 Production by Plaque Macrophages

Blood monocytes from patients undergoing carotid and noncarotid surgery produced small amounts of IL-1 spontaneously *in vitro* (Figure 2). In the carotid endarterectomy group, blood monocyte IL-1 production was 0.27 ± 0.18 ng/10^6 cells/24 hours (n = 12), and in the noncarotid group 0.32 ± 0.19 ng/10^6 cells/24 hours (n = 4). Macrophages isolated from atheromatous plaques tended to produce less IL-1 than blood monocytes. Production of IL-1 by macrophages from carotid plaques was undetectable in 10 supernatants. Mean IL-1 production for carotid plaque macrophages was 0.020 ± 0.015 ng/10^6 cells/24 hours (n = 13) (P = 0.075 compared with blood monocytes). In the noncarotid group, five of seven supernatants had undetectable IL-1 production (mean 0.004 ± 0.003 ng/10^6 cells/24 hours).

Cell lysates of macrophages from carotid (n = 14) and noncarotid (n = 6) plaques contained no detectable IL-1. Lysates of blood monocytes from endarterectomy patients contained detectable IL-1. In the carotid group, cell-associated IL-1 in blood monocytes was 0.06 ± 0.03 ng/10^6 cells (n = 14), and in the noncarotid group cell-associated IL-1 was 0.16 ± 0.07 ng/10^6 cells (n = 6). These values were not significantly different.

Macrophage Cytokine Expression within Tissue Sections

Serial sections of atheromatous plaques were evaluated using monoclonal antibodies for the presence of macrophages (CD14), smooth muscle cells (actin), and TNF and IL-1 (Table 1); results are shown for representative carotid (Figure 3a–d) and noncarotid (Figure 3e–h) specimens. In general, plaques consisted of >80% $CD14^+$ macrophages (Figure 3b and 3f) plus lesser numbers of lymphocytes and

Figure 3. *Immunoperoxidase labeling of serial sections from atheromatous plaques from carotid endarterectomy* (a–d) *and femoral artery* (e–h) *specimens. The photomicrograph demonstrate macrophage rich regions where less than 5% of cells were stained for the smooth muscle specific actin* (a,e). *The majority (>80%) of plaque cells expressed the macrophage antigen CD14* (b,f). *Macrophages within carotid plaques showed labeling for TNF,* (c), *whereas essentially no staining was seen in most noncarotid specimens* (g). *Only faint or no staining for IL-1 was detected in either carotid or noncarotid specimens* (d,h). *(Cryostat sections, 4 layer immunoperoxidase, hematoxylin counterstain, ×400– original magnification).*

only small numbers (5–10%) of actin-positive smooth muscle cells (Figure 3a and 3c). All seven carotid specimens showed cytoplasmic and surface expression of TNF on cells in macrophage-rich regions. Intensive labeling (3+) for TNF was present in four of the specimens (Figure 3c). By comparison, six of the seven noncarotid specimens showed minimal or no labeling (0–1+) for TNF, and only one specimen showed moderate labeling (2+). Only trace or no labeling for IL-1 was detected in any of the carotid specimens (Figure 3d), and IL-1 was detected in only one noncarotid specimen. The latter specimen was the only noncarotid specimen that showed detectable TNF labeling (3+). No staining of these specimens occurred using isotype-matched control monoclonal antibodies, whereas dense labeling for IL-1 and TNF was found using control lipopolysaccharide-treated monocyte cell smears.

Discussion

Macrophages have the potential to play a prominent role in the initiation and progression of atherosclerotic plaques via their ability to produce a variety of pro-inflammatory cytokines, including TNF and IL-1. TNF has been demonstrated by immunohistochemistry on macrophages, smooth muscle cells, and endothelial cells in atheromatous vessels from postmortem and amputation specimens.[19]

Intimal smooth muscle cells in atherosclerotic plaques have been shown to express cell surface TNF and TNF mRNA.[20] Cultured vascular smooth muscle cells also produce TNF mRNA and product[21] and IL-1 mRNA[22] in response to endotoxin. IL-1α and -β mRNA has been demonstrated in foam cells, smooth muscle cells, and endothelium in atherosclerotic plaques in monkeys.[23] The capacity of plaque macrophages to produce cytokines without exogenous stimulation or prolonged culture has not been previously reported.

In the current studies, macrophages were isolated from atherosclerotic plaques removed during vascular procedures performed in patients with clinically significant atherosclerotic disease. Their spontaneous production of cytokines TNF and IL-1 was studied *in vitro*. For comparison, the cytokine production of blood monocytes isolated concurrently from these patients was also assessed.

The isolation technique used for plaque macrophages is rapid and yields a relatively pure population of viable macrophages suitable for functional studies. This technique, previously used to demonstrate augmented tissue factor expression by macrophages from atherosclerotic carotid plaques,[6] involves mechanical disruption, gentle teasing of the tissue to separate cells, and adherence to plastic to purify macrophages. Macrophages, clearly identified by several morphological criteria, were obtained with minimal handling in 1 hour, in sufficient numbers to allow assessment of spontaneous cytokine production in short-term serum-free culture.

Since it is possible that this technique selects a subpopulation of plaque macrophages that may not be representative of plaque macrophages as a whole, immunohistological demonstration of cytokines on plaque macrophages "*in situ*" was also performed.

The results show selective augmentation of cytokine production by cells in macrophage-rich regions of carotid plaques. Some minor contribution of smooth muscle cells to TNF production by isolated macrophage populations cannot be entirely excluded. Three pieces of evidence suggest that increased TNF production by macrophages from carotid plaques does not result from the isolation procedure. First, *in situ* staining studies confirm increased TNF expression on macrophages in carotid plaques. Second, the same isolation procedure does not activate noncarotid macrophages, and third, atheromatous material from carotid plaques does not augment TNF production by blood monocytes.

Macrophages from carotid plaques demonstrated augmented production of TNF *in vitro* compared to blood monocytes. TNF release by carotid macrophages was significantly greater and cell associated TNF was also greater than in blood monocytes. This finding was further supported by *in situ* immunohistochemical localization of TNF in atherosclerotic plaques. TNF was demonstrated on the surface membrane and in the cytoplasm of cells in macrophage-rich regions (identified in serial sections using validated a monoclonal antibody) of carotid plaques.

TNF release and cell-associated TNF of macrophages from atherosclerotic plaques in the distal aorta or femoral arteries were not augmented. The reasons for this are not clear; however, it may reflect different inflammatory activity in plaques from carotid and distal aortic and femoral sites at the time of surgery. Half of the patients undergoing carotid surgery had recent cerebral thromboembolic symptoms, suggesting active inflammatory disease. Since macrophages from aortic and femoral

plaques did not exhibit augmented TNF production, it is unlikely that augmented TNF expression by carotid plaque macrophages is merely a reflection of a general maturation of blood monocytes into tissue macrophages.

IL-1 production by plaque macrophages from carotid or distal aortic and femoral sites was undetectable in the majority of cases. Cell-associated IL-1 was also undetectable in plaque macrophages. Small amounts of IL-1 were produced by blood monocytes from these patients, but the levels were not significantly different from those of plaque macrophages. Cell lysates from blood monocytes also contained IL-1, although less than was released in culture. The demonstration of unreleased IL-1 in blood monocytes, but not in macrophages, is consistent with previous observations.[24] Together these results demonstrate a lack of up-regulation of IL-1 production by macrophages in atheromatous plaques, in contrast to TNF production.

The stimuli-activating macrophages in atheromatous plaques are unknown. A number of potential activators, including T lymphocytes and lipoproteins, are prominent within plaques. Endotoxin is also a potent macrophage activator that can contaminate cultures. In the current studies, endotoxin levels were undetectable in the *Limulus* amebocyte lysate assay in macrophage supernatants after culture.

In conclusion, these studies demonstrate augmented TNF production without augmentation of IL-1 production by macrophages from carotid plaques. They provide evidence of selective activation of macrophage inflammatory functions and further support an active pro-inflammatory role for macrophages in atheroma.

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